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REGULAR ARTICLE

Factors influencing the release of the biological nitrification inhibitor 1,9-decanediol from rice (*Oryza sativa* L.) roots

Xiaonan Zhang · Yufang Lu · Ting Yang · Herbert J. Kronzucker · Weiming Shi

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Abstract

Aims Root exudates of rice (*Oryza sativa* L.) can inhibit nitrification in *Nitrosomonas* bioassays, and 1,9-decanediol was recently identified as an important new biological nitrification inhibitor (BNI) from rice. However, the release characteristics of 1,9-decanediol have not been studied. The present study was designed to identify the major factors influencing the release of 1,9-decanediol from rice roots.

Methods Rice plants were hydroponically grown in controlled environment chambers for 6 weeks, and root exudates were collected. Responses of exudate release to nitrogen form and concentration, pH, aeration, and bacterial inoculation were explored. The pH of root exudates, collected under different nitrogen-provision regimes, was determined, and 1,9-decanediol levels in exudates were monitored.

Results Ammonium (NH_4^+) and low pH in the root environment stimulated the release of 1,9-decanediol from rice roots. When only a part of the root system was exposed to NH_4^+, the secretion of 1,9-decanediol was triggered in the whole root system. Aeration of the root environment significantly enhanced 1,9-decanediol release. The presence of two major nitrifiers (*Nitrosomonas europaea* and *Nitrosomonas stercoris*) in the root medium stimulated release of 1,9-decanediol, whereas denitrifiers had no effect.

Conclusions Our results demonstrate that the release of 1,9-decanediol is enhanced by low to moderate concentrations of NH_4^+ (≤1.0 mM), low pH, and aeration of the rhizosphere. Our study provides the first evidence of significant 1,9-decanediol secretion induced by nitrifying bacteria.

Keywords Biological nitrification inhibition/inhibitor (BNI) · 1,9-decanediol · Rice (*Oryza sativa* L.) · Ammonium · Nitrifiers

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Introduction

Nitrification, which converts ammonium (NH_4^+) / ammonia (NH_3) to nitrate (NO_3^-), is one of the key components of the global nitrogen (N) cycle and greatly contributes to the loss of fertilizer N, by facilitating the processes of leaching and denitrification (Raun and Johnson 1999; Subbarao et al. 2006b). The roots of certain plants release biological nitrification inhibitors (BNIs) that suppress bacterial nitrification in soils,

thereby increasing nitrogen-use efficiency (NUE) and presenting a new strategy to curb agricultural N losses. Since the first report of biological nitrification inhibition (BNI) in *Brachiaria humidicola*, BNIs have been discovered in several crops, including sorghum (*Sorghum bicolor* L.), pearl millet (*Pennisetum glaucum* L.), groundnut (*Arachis hypogaea* L.), and wheat (*Triticum aestivum* L.) (Coskun et al. 2017a, b; O'Sullivan et al. 2016; Subbarao et al. 2006a, 2007a). More recently, the major cereal crop rice (*Oryza sativa* L.) was also reported to produce detectable BNI activity in root exudates (Tanaka et al. 2010). We previously isolated 1,9-decanediol as the first BNI identified from rice root exudates. The chemical was shown to specifically block the ammonia monooxygenase (AMO) step in bacterial ammonia oxidation. Quantities of 1,9-decanediol in root exudates and BNI abilities correlated positively with ammonium-use efficiency and ammonium preference in rice (Sun et al. 2016b). However, the factors influencing the release of 1,9-decanediol from rice roots are unknown.

Several studies have documented that several abiotic factors can affect the production and release of root exudates, and chief among these are nutrient status and pH (Bowsher et al. 2015; Carvalhais et al. 2011; Dayan 2006; Khorassani et al. 2011). The release of BNI compounds from plant roots is regulated by nitrogen availability in the root environment, presumably reflecting a strategy to conserve available N in the reduced form (Subbarao et al. 2007c; Zakir et al. 2008). Recent research has demonstrated that both N form and concentration in the root environment are critical to the sustained synthesis and release of BNIs in the roots of pasture grasses, sorghum, and wheat (Subbarao et al. 2007b, c, 2009; Zakir et al. 2008; Zeng et al. 2016). For example, secretion of brachialactone from *B. humidicola* roots is enhanced only in the presence of NH_4^+ and not NO_3^-. (Subbarao et al. 2009). The release of BNIs from sorghum roots was increased at NH_4^+ concentrations below 1.0 mM (Zeng et al. 2016). In addition, a localized response was reported as the release of BNIs was triggered only in the part of the root system exposed to NH_4^+ in *B. humidicola* and sorghum (Subbarao et al. 2009; Zhu et al. 2012). However, in rice roots, whether the release of 1,9-decanediol is influenced by the plant's N status, including N form and concentration, and localization in the root environment is unclear.

Ammonium uptake by root cells is known to depolarize the plasma-membrane electrical potential and increase net H^+ release, leading to acidification of the rhizosphere (Schubert and Yan 1997; Wang et al. 1993). Thus, secondary acidification effected by NH_4^+ uptake on BNI release must be considered. In the roots of *B. humidicola*, NH_4^+ and low pH in the root zone together have a synergistic effect on BNI-compound release (Subbarao et al. 2007c). Similarly, the release of hydrophilic BNIs from sorghum roots is stimulated by rhizosphere pH <5.0, while the dependence of the release of hydrophobic BNIs on rhizospheric pH depends on genotype (Di et al. 2018; Subbarao et al. 2013). However, how the release of 1,9-decanediol responds to pH in rice roots is unknown.

The degree of oxygenation of the root rhizosphere is essential for plant root function, and is of special importance in rice, which typically grows in flooded, hypoxic to anoxic environments (Kronzucker et al. 1998). Both soil aeration and nutrient solution aeration increase rhizospheric oxygen content, significantly enhancing metabolic root activity in general and increasing the plant's ability to engage detoxification mechanisms in the root system (Niu et al. 2012; Yuan et al. 2015). The presence of oxygen dramatically affects the efficiency of cellular adenosine triphosphate (ATP) production in root cells and is required in numerous cellular pathways, including heme, sterol and fatty-acid biosynthesis (Geigenberger 2003). Meanwhile, the transport of a very broad range of substrates (metabolic products, ions, lipids, and xenobiotics) is driven by membrane-bound transport proteins, using the energy from ATP hydrolysis (Weston et al. 2012). Thus, any discussion of the factors governing the release of 1,9-decanediol from rice roots must take into account oxygen levels in the root environment.

In addition to the abiotic environmental factors listed above, biotic factors must also be considered. Plant roots and soil bacteria are engaged in a plethora of interactions, some of which involve highly specific forms of chemical communication (Badri and Vivanco 2009; Chagas et al. 2018; Paterson et al. 2007). De-la-Pena et al. demonstrated that the presence of microbes modifies the composition of proteins present in root exudates and that a given plant can modulate the exudation of proteins by a given bacterial strain (De-la-Pena et al. 2008). In the soil N cycle, the role of the Rhizobiaceae is of special importance, in that its members form symbiotic associations with leguminous plants to fix

atmospheric nitrogen. Flavonoids and betaines released by leguminous roots are perceived by rhizobia and lead to the release of Nod factors, which cause root hairs to curl, providing a haven for bacterial colonizers (Gage 2004; Subramanian et al. 2007). Similarly, microorganisms involved in both nitrification and denitrification might play important roles in the N cycle. Specifically, 1,9-decanediol from rice root exudates can act on the AMO pathway of *Nitrosomonas europaea* to inhibit nitrification. However, the influence of microbes on BNI secretion from plants has not, to date, been studied. Thus, we explored whether the presence of major nitrifiers or denitrifiers can affect the secretion of 1,9-decanediol from rice roots.

In the present study, different forms and concentrations of nitrogen, pH, aeration, and bacterial inoculants were applied to trap solutions to investigate their role in the release of 1,9-decanediol from rice roots. The goal of the study design was to develop new insights into our understanding of the characteristics of the secretion of 1,9-decanediol, the first BNI identified in root exudates of rice, the world's most important crop species.

Materials and methods

Experiment 1: Cultivation of rice plants

The variety of rice (*Oryza sativa* L.) used in this study was WYJ7 (Wuyunjing7). Seeds of rice were sterilized with 10% H_2O_2 for 30 min, rinsed, and then soaked with distilled water for 24 h. The seeds were then germinated on floating nets in a culture box containing 0.5 mM $CaCl_2$. After 3-d incubation at 30 °C in the dark, the germinated seeds were placed under light (100 μmol m^{-2} s^{-1}) for 1 week to prevent the transpiration caused by high light intensity, and the $CaCl_2$ solution was replaced with half-strength modified Kimura-B nutrient solution. Subsequently, three 10-d-old seedlings at a time were bundled and transplanted into a larger culture box with an identical nutrient solution. Then, plants were placed under high light intensity (400 μmol m^{-2} s^{-1}) provided by high-pressure sodium lamps. Nutrient solution composition and management of culture solutions were as described previously (Sun et al. 2016b). Composition was as follows, in mM: 0.5 NH_4NO_3; 0.18 KH_2PO_4; 0.54 $MgSO_4{\cdot}7H_2O$; 0.18 KCl; 0.36 $CaCl_2$; 2×10^{-4} $CuSO_4{\cdot}5H_2O$; 5×10^{-4} $MnCl_2{\cdot}4H_2O$; 4×10^{-4} $ZnSO_4{\cdot}7H_2O$; 3×10^{-4} H_3BO_3; 1×10^{-4} $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$; 2×10^{-2} Na_2EDTA-Fe. Two weeks after sowing, the nutrient solution was changed to full strength. The pH of the solution was 5.8, and 0.2 g L^{-1} MES was added to maintain the pH during cultivation. The nutrient solution was changed every 2 days, and the solution volume was restored daily with deionized water. The plants were grown in a controlled-environment chamber with a day/night temperature regime of 28 °C/25 °C, 65% humidity, a 14/10-h light/dark photoperiod. Rice seedlings were grown for 6 weeks prior to collection of root exudates.

Experiment 2: Root exudate collection

Experiment 2a: Influence of nitrogen form, ammonium concentration, and pH in the root zone on 1,9-decanediol release from rice roots

30 six-week-old seedlings for each replicate ($n = 3$) were rinsed consecutively with deionized water before use. The seedlings were then transferred into a tall-form glass beaker, and the roots were immersed gently in 1 L of 1.0 mM NH_4Cl or 1.0 mM KNO_3 solution, with 1 L of Milli-Q water as the control, to detect the influence of N form on 1,9-decanediol release. To investigate the effect of ammonium concentration on the release of 1,9-decanediol, rice root exudates were collected in solutions containing NH_4Cl of different concentration (0, 0.1, 0.5, 1.0, 3.0, 6.0 mM). Meanwhile, to investigate the effect of pH on 1,9-decanediol release, the pH of the collection solutions (without nitrogen) was adjusted to 3.0, 5.8, and 7.0 separately, by using either 1.0 M NaOH or HCl (Zhu et al. 2012). 1 mL 0.1 M $CaCl_2$ was added into all collection solutions. For longer collection periods exceeding 2 h, low concentrations of Ca are necessary to limit osmotic stress and possible passive leakage and/or diffusion (Schapire et al. 2009). The shoots of rice seedlings were held and supported with sterilized sponges, and the beakers were wrapped in tinfoil to protect roots from light. Mechanical damage to roots can lead to a significant alteration of both exudate amount and composition. Therefore, extreme attention was paid during manipulation. Water was replenished after 12 h, to avoid excessive evapotranspiration. After 24 h, both shoots and roots were washed, separated and freeze-dried for weighing. After collecting root exudates with different concentration of ammonium for 24 h, the pH value of root exudate solutions was measured using

a pH meter (S400 SevenExcellence™, Mettler Toledo, Shanghai, China).

Experiment 2b: Influence of localized NH_4^+ supply on the release of 1,9-decanediol in a split-root system

This experiment was performed as described by Subbarao et al., with some modifications (Subbarao et al. 2009). Rice plants were raised hydroponically with NH_4NO_3 as the sole N source. After 3 weeks of growth, the root system of each plant was divided in half, and each half was grown in a separate nutrient tank. Nine plants were transplanted to a split-root system, which was also wrapped in tinfoil to protect roots from light exposure. After 3 weeks of separate growth, the collection solutions from each tank were as follows: a. Milli-Q water: Milli-Q water; b. Milli-Q water: 1.0 mM NH_4Cl; c. 1.0 mM NH_4Cl: 1.0 mM NH_4Cl. Additionally, $CaCl_2$ was added to both sides of the apparatus to provide a final concentration of 0.1 mM. After 24 h, root exudates were collected separately. Then, root exudates from 3 tanks (about 400 ml for each) were merged together to obtain about 1.2 L of collected solutions, which could then satisfy the quantitative analysis for 1,9-decanediol. The experiment was repeated three times. Both shoots and roots were washed, separated, and freeze-dried for weighing.

Experiment 2c: Effect of aerating the collection solution on the release of 1,9-decanediol

The root exudate collection process was as above. After rice seedlings (30 6-week-old seedlings for each replicate; $n = 3$) were removed from hydroponics, and their roots were gently rinsed with ultrapure water. Aeration was supplied by means of a bubble stone connected to an air supply to provide gentle bubbling in the collection solution containing 0.1 mM $CaCl_2$ (Air pump, 8 W, 1 × 3 L/min). This breaks the surface tension of the liquid and injects air directly into the solution. The same solutions without aeration were used for exudate collection in the control group. Water was replenished after 12 h to avoid excessive evapotranspiration. After 24 h, both shoots and roots were washed, separated, and freeze-dried for weighing.

Experiment 2d: Influence of nitrifying and denitrifying bacteria on 1,9-decanediol release

The nitrifying bacteria, *Nitrosomonas europaea* (NBRC 14298) and *Nitrosomonas stercoris* (NBRC 110753) were obtained from the NITE Biological Resource Center (NBRC), Tokyo, Japan. The two strains were cultured under the conditions described by Sun et al. (2016b). *Nitrosomonas europaea* and *Nitrosomonas stercoris* were both grown aerobically in HEPES medium, as recommended by NBRC, containing the following nutrients (1 L): $(NH_4)_2SO_4$, 2.5 g; KH_2PO_4, 0.5 g; HEPES, 11.92 g; $NaHCO_3$, 0.5 g; $MgSO_4{\cdot}7H_2O$, 100 mg; $CaCl_2{\cdot}2H_2O$, 5 mg; Fe-EDTA, 75 mg; pH 7.8–8.0. Bacteria were cultured in 500-mL flasks containing 200 mL of HEPES medium using an incubation shaker (set at 200 rpm, and 30 °C). The denitrifying bacterial strain *Pseudomonas fluorescens 01047* was obtained from the Agricultural Culture Collection of China (ACCC), and *RWX31*, identified as *Pseudomonas sp*, was isolated from 53 different denitrifying bacterial cultures, as it had the highest denitrification efficiency (Zhou et al. 2013). The two strains were both cultured in LB medium containing the following nutrients (1 L): Tryptone, 10 g; Yeast, 5 g; NaCl, 10 g; pH 7.0. Before collecting root exudates, a 7-d-old culture mix of the two strains of nitrifiers and a 24-h-old culture mix of the two strains of denitrifiers were centrifuged (6500 rpm for 20 min), washed twice with sterile medium, and mixed with freshly prepared rice root exudate collection solutions, to achieve a final OD_{600} of 0.02 (De-la-Pena et al. 2008; Walker et al. 2004). Similarly, 30 6-week-old rice plants for each replicate ($n = 3$) were used to collect root exudate as described above. After 24 h, both shoots and roots were washed, separated, and freeze-dried for weighing.

Experiment 3: Pretreatment of root exudates and 1,9-decanediol analysis

The collected exudates were pretreated immediately or stored at 4 °C until extracting within 3 days, according to the method described before (Sun et al. 2016a). The collection solutions were filtered using 0.45-μm and 0.22-μm filter membranes to remove pieces of roots and microorganisms. Then, the filtered solutions were subjected to a solid-phase extracting system (C18 SPE columns, 17% carbon content, 1 g/6 mL, CNW) to retain 1,9-decanediol in root exudates. Finally, the

residue was dissolved in 10 mL of HPLC-grade methanol and stored in an amber vial under −20 °C. Four milliliters of root exudate samples (in methanol) of 6-week-old seedlings were evaporated to dryness under N_2 and derivatized with 200 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 60 °C for 0.5 h. The mixture was then evaporated to dryness again, dissolved in 200 μL of hexane and subjected to GC. GC analysis was performed on an Agilent 7890 chromatograph equipped with a fused silica capillary column HP-5 (25 m × 0.2 mm × 0.33 μm) and a flame ionization detector (FID). Splitless injection was performed at 250 °C; the oven temperature was initially 80 °C and was increased to 250 °C, at a rate of 20 °C min^{-1}, and then to 300 °C, at a rate of 6 °C min^{-1}. Helium was used as the carrier gas, provided at a flow rate of 1.0 mL min^{-1}, and the sample size was 2 μL. Authentic 1,9-decanediol was used to produce a standard curve.

Statistical analysis

The experimental data were subjected to the SPSS Statistics 18.0 software (Chicago, IL, USA). One-way analysis of variance (ANOVA) was performed on all the data, to confirm the variability of data and validity of the results. All the figures were drawn by Origin 8.1 software (Origin Lab, USA). Differences between the means among treatments were compared using Duncan's multiple-range test at 0.05 probability levels.

Results

Effect of nitrogen form and ammonium concentration on the release of 1,9-decanediol

Rice seedlings were grown hydroponically for 6 weeks with NH_4NO_3 as the N source prior to collection of root exudates. Our results show that different N forms in the trap solution exert a significant ($P < 0.05$) influence on the secretion of 1,9-decanediol from rice roots. The addition of NH_4^+ to exudate solutions enhanced 1,9-decanediol release, at rates 2.4 times higher than under control (collected with distilled water, with Ca^{2+}). However, 1,9-decanediol release did not change significantly with additions of NO_3^- (Fig. 1a). In addition, treatment with low to medium concentrations (up to 1.0 mM) of NH_4^+ increased the release of 1,9-decanediol, and rates of release tripled at 1.0 mM compared to control. However, at higher concentrations (1.0 to 6.0 mM) of NH_4^+, this effect became mild to insignificant (Fig. 1b). The results show that only NH_4^+ has a stimulatory effect on the 1,9-decanediol release, that this effect is strong but most pronounced at low concentrations, and that NO_3^- does not impact release.

Influence of trap solution pH on the release of 1,9-decanediol

After collecting root exudates under different concentrations of ammonium for 24 h, the pH was measured using a pH meter. Results show that the pH of the collection solutions was significantly ($P < 0.05$) different among treatments. As expected, the uptake of NH_4^+ strongly acidified the root exudate solution, and the pH of the collection solutions ranged from 2.4 to 3.8, while the presence of NO_3^- caused a significant increase of the pH compared with that of root exudates collected with distilled water (Treatment 0; Fig. 2a). To investigate whether the increase of 1,9-decanediol release was only caused by the decline of pH after adding NH_4^+ to the collection medium, different pH treatments were performed when collecting root exudates. The results show that only the low pH treatment (pH 3.0, adjusted with 1.0 M HCl) caused approximately a doubling of the amount of 1,9-decanediol release compared with the control, while the two other treatments (pH 5.8, 7.0, adjusted with 1.0 M NaOH) showed no statistically significant difference ($P < 0.05$) (Fig. 2b). The results indicate that both NH_4^+ and low pH in the root environment can stimulate 1,9-decanediol release from rice roots, and that this effect is additive.

Influence of localized NH_4^+ supply on the release of 1,9-decanediol in a split-root system

To test the idea that certain parts of the root system exposed to ammonium might induce responses in other parts, a split-root system was designed to collect root exudates from separate root chambers for the same plants (Fig. 3a). When both sides of the rice root system in the split-root design were exposed to NH_4^+, the amount of 1,9-decanediol in separate tanks (~550 ng g^{-1} root DW d^{-1}), or the total 1,9-decanediol release in the whole system, were all significantly higher

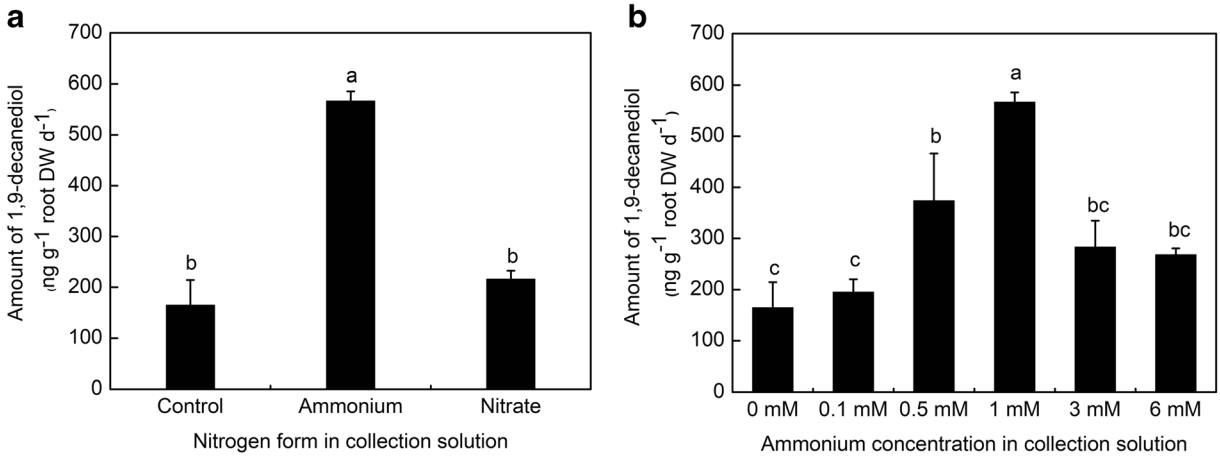

Fig. 1 Influence of N form (i.e. 1.0 mM N as NH_4^+ vs. NO_3^-) (**a**) and NH_4^+ concentration (**b**) in root exudate collection solutions on 1,9-decanediol release in rice grown hydroponically for 6 weeks with NH_4NO_3 as N source. Vertical bars indicate ±SE (standard error) of three replications. Lower-case letters represent statistical differences (Duncan's test, at $P < 0.05$)

than in the control with distilled water (with Ca^{2+}) on both sides. However, when trap solutions were different on the two sides of the split-root system, the release of 1,9-decanediol on the NH_4^+ side was increased to almost the same level as that of root exudates collected with NH_4^+ on both sides; while the secretion of 1,9-decanediol was 342 ng g^{-1} root DW d^{-1} in the part where the root system was not supplied with NH_4^+, which was, however, much higher than in the control with distilled water in both sides (Fig. 3b). Thus, release of 1,9-decanediol was triggered not only in the part of the root system exposed to NH_4^+ but also in the entire root system.

Influence of aeration in collection solution on the release of 1,9-decanediol

In the normal protocol, root exudate collection occurred in distilled water and without extra aeration during the collecting period. Under this condition, the secretion of 1,9-decanediol from rice root was 216 ng g^{-1} root dry weight d^{-1}. However, when the collection solution was sufficiently aerated with an air pump, the release of 1,9-decanediol was increased by 63%, and it showed significant ($P < 0.05$) difference compared to the control (Fig. 4). This indicates that sufficient aeration is important for the ability of rice roots to secrete 1,9-decanediol.

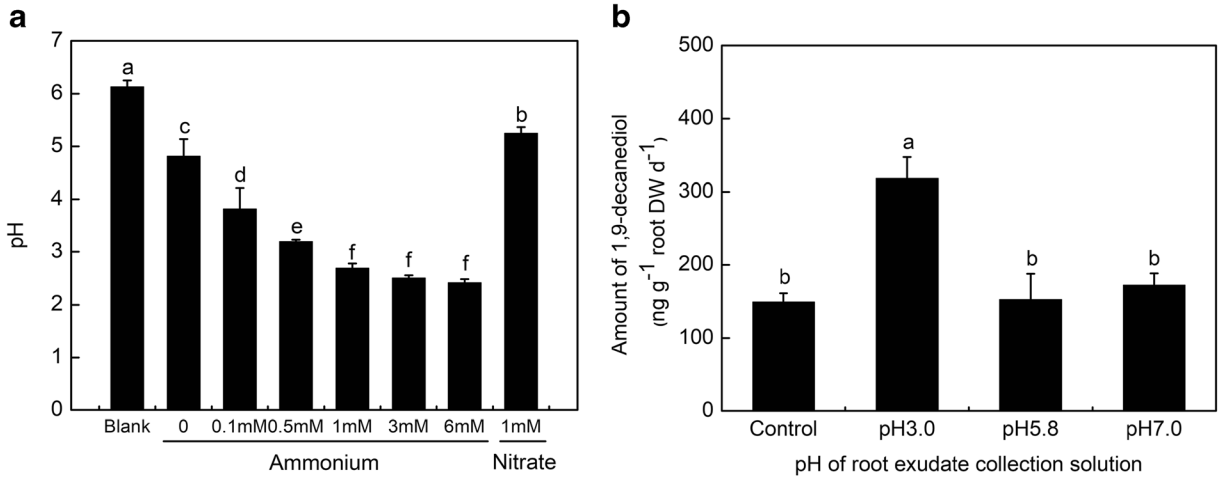

Fig. 2 pH of root exudate collection medium after 24 h (**a**), and effect of root exudate collection solution pH (solution pH 3.0, 5.8, and 7.0) on 1,9-decanediol release in rice grown hydroponically for 6 weeks with NH_4NO_3 as N source (**b**). Vertical bars indicate ±SE (standard error) of three replications. Lower-case letters represent statistical differences (Duncan's test, at $P < 0.05$)

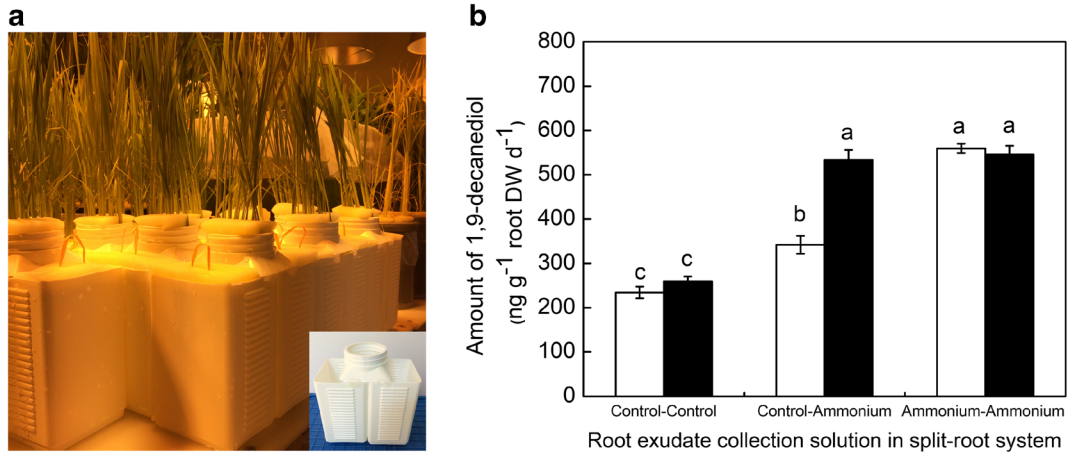

Fig. 3 Split-root experimental system used for collecting root exudates from rice (**a**) and influence of ammonium on 1,9-decanediol release from rice roots in a split-root system (**b**). After 6 weeks of growth, collection solutions of each tank in every system were as follows: a. Milli-Q water: Milli-Q water; b. Milli-Q water: 1.0 mM NH_4Cl; c. 1.0 mM NH_4Cl: 1.0 mM NH_4Cl. Additionally, 0.1 M $CaCl_2$ was added into both sides of this apparatus to produce a final concentration of 0.1 mM. Vertical bars indicate ±SE (standard error) of three replications. Lower-case letters represent statistical differences (Duncan's test, at $P < 0.05$)

Influence of nitrifying and denitrifying bacteria on 1,9-decanediol release

Our results show that the presence of ammonium-oxidizing bacteria, *Nitrosomonas europaea* and *Nitrosomonas stercoris*, in the collection solution can significantly ($P < 0.05$) enhance the secretion of 1,9-decanediol from rice roots, from 170 ng g^{-1} root dry weight d^{-1} to nearly 390 ng g^{-1} root dry weight d^{-1} (Fig. 5), more than doubling the rate of release after the addition of the two strains of *Nitrosomonas*. However, the presence of two strains of denitrifying bacteria, *Pseudomonas fluorescens 01047* and *RWX31*, in the trap solutions showed no significant effect on 1,9-decanediol secretion compared with the control collected with distilled water (Fig. 5).

Discussion

Plant root exudates can profoundly modify soil microbial communities and influence their N transformations (Coskun et al. 2017a). In recent decades, some compounds exuded from plant roots have been shown to effectively inhibit nitrification in the rhizosphere (Subbarao et al. 2006a, 2009, 2013; Tanaka et al.

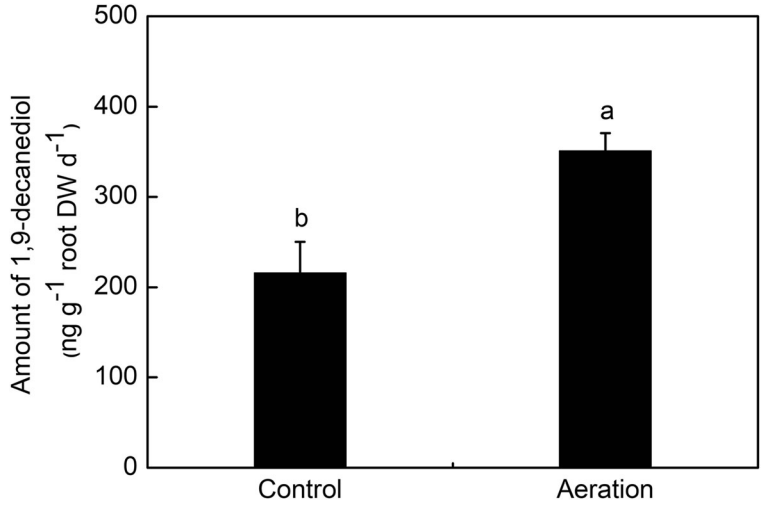

Fig. 4 Amount of 1,9-decanediol in rice root exudates collected with different conditions of aeration. Vertical bars indicate ±SE (standard error) of three replications. Lower-case letters represent statistical differences (Duncan's test, at $P < 0.05$)

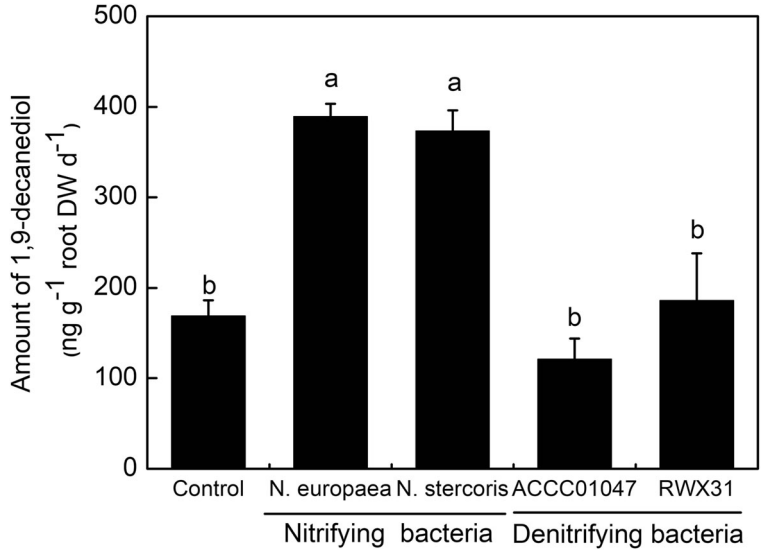

Fig. 5 The release of 1,9-decanediol from the roots of rice grown with NH_4NO_3 as the N source. Root exudates were collected with nitrifying bacteria (*N.europaea*: *Nitrosomonas europaea,* and *N. stercoris: Nitrosomonas stercoris*) and denitrifying bacteria (*ACCC 01047*: *Pseudomonas fluorescens 01047*, and *RWX31*: *Pseudomonas sp*). Vertical bars indicate ±SE (standard error) of three replications. Lower-case letters represent statistical differences (Duncan's test, at $P < 0.05$)

2010; Zakir et al. 2008). We previously reported that 1,9-decanediol identified from rice root exudates blocks the AMO pathway of ammonia oxidation and its secretion is positively correlated with rice ammonium-use efficiency and ammonium preference (Sun et al. 2016b). However, the factors affecting the release of 1,9-decanediol have remained unclear.

Our results obtained here provide evidence that the release of 1,9-decanediol is promoted by the presence of NH_4^+ rather than NO_3^- in the rice root environment. Rice normally grows in flooded soils high in NH_4^+ (Kronzucker et al. 1999, 2000; Kirk and Kronzucker 2005) with only minor presence of NO_3^- but is able to use both N sources (Kronzucker et al. 1999; Kirk and Kronzucker 2005). In the NH_4Cl root exudate treatments, 1,9-decanediol release was about 2.4-fold higher than in the control (Fig. 1a). Interestingly, the presence of NH_4^+ in the root zone showed a concentration-dependent stimulatory effect on the 1,9-decanediol release, and the amount of 1,9-decanediol was highest at lower to intermediate concentrations of NH_4^+, with a peak at 1.0 mM, rather than at higher concentration (1.0 to 6.0 mM) (Fig. 1b). This concentration range is what is expected under field conditions in rice paddies (Kronzucker et al. 1998; Wang et al. 1993). It indicates that the BNI attributes of 1,9-decanediol in rice is also a regulated attribute and is associated with the availability of N in the root environment. This is broadly consistent with previous studies that NH_4^+ triggers the release of BNIs in the roots of pasture grasses, sorghum, and rice (Subbarao et al. 2007c; Tanaka et al. 2010; Zakir et al. 2008; Zhu et al. 2012).

We found that the stimulatory role played by NH_4^+ in 1,9-decanediol secretion partially resulted from the low pH in the root environment. The presence of ammonium in the trap solutions significantly decreased the pH of the collection solutions (Fig. 2a). This is a well-known phenomenon, caused by the uptake and assimilation of NH_4^+, which is associated with depolarization of the plasma-membrane electrical potential, increasing net proton release and resulting in acidification of the rhizosphere, often rapidly and dramatically, depending on the rates of NH_4^+ transport and metabolism (Di et al. 2018; Schubert and Yan 1997; Zhu et al. 2009; Zeng et al. 2012). Our study indicates that the release of 1,9-decanediol was indeed influenced by the pH in the trap solutions, and that low pH (with a peak at pH 3.0) can increase its release significantly, albeit to much lower peaks than those seen with NH_4^+ treatment (Fig. 2b). Thus, we speculated that the stimulation by NH_4^+ (at concentrations up to 1.0 mM) of 1,9-decanediol release might, at least in part, be due to the acidification induced by its uptake and assimilation, while high concentrations of NH_4^+ (3.0 and 6.0 mM) might induce ammonium toxicity, with concomitant stresses on the membrane system of the rice root (Britto and Kronzucker 2002; Duan et al. 2006; Van den Berg et al. 2005). It is generally agreed that, in plant cells, H^+ is pumped out by plasma membrane H^+-ATPases, and that plasma membrane H^+-ATPase activity is activated by NH_4^+ nutrition and low rhizosphere pH (Britto and

Kronzucker 2005), as shown in the roots of barley, rice, and sorghum (Di et al. 2018; Yamashita et al. 1995; Zhu et al. 2009; Zeng et al. 2012). The key function of this enzyme is to generate an H^+-electrochemical gradient, thereby providing the driving force for the active influx and efflux of ions and metabolites across the plasma membrane (Palmgren and Harper 1999; Zeng et al. 2016). It is possible that 1,9-decanediol release is facilitated by the protonation of, and transport through, a voltage-dependent anion efflux channel, and its release might, thus, be closely linked to the regulation of PM H^+-ATPases, similar to what has been suggested for BNI release in sorghum (Zhu et al. 2012). The direct influence of NH_4^+ itself on the release of 1,9-decanediol might be because endogenous NH_4^+ may serve as an allosteric regulator of the activities of the enzymes of 1,9-decanediol synthesis, as well as providing a signal to the plasma membrane for its release (Liu and von Wiren 2017; Zakir et al. 2008). Such mechanistic relationships at the metabolic level will have to be explored in the future.

The stimulatory effect of NH_4^+ on 1,9-decanediol excretion was also confirmed in our split-root experiments, where release of 1,9-decanediol was significantly higher in the part of the root system supplied with (1.0 mM) NH_4^+ compared with distilled water (Fig. 3b). Similarly, previous studies have shown that release of BNI compounds, including the cyclic diterpene called brachialactone, from *B. humidicola* as well as sorghum, was stimulated by NH_4^+ (compared to NO_3^-) in a split-root system (Subbarao et al. 2009; Zhu et al. 2012). However, it is noteworthy that, in our study, local availability of ammonium for roots promoted the secretion of 1,9-decanediol as well in other parts of the root system (Fig. 3b). This effect of NH_4^+ on 1,9-decanediol release is probably associated with a signaling rather than a simple nutritional effect. Perhaps the most plausible mechanistic view is that, once perceived, ammonium signals eventually affect a set of genes and/or proteins involved in the synthesis of 1,9-decanediol as well as its release from roots. As described in several comprehensive reviews, when only a part of the root system is exposed to elevated NH_4^+, especially in waterlogged and acidic soils, such as those rice plants typically grow in, plants induce NH_4^+ uptake and detoxification mechanisms in other parts of the root system; induction of local responses only in certain parts of the root system will then rely on the coordination of internal and external ammonium-dependent signals (Giehl et al. 2014; Liu and von Wiren 2017).

Our results demonstrate that the presence of NH_4^+ and the physiological consequences associated with its uptake in the root zone appear to play stimulatory roles in 1,9-decanediol release from rice roots, indicating that 1,9-decanediol secretion is also an adaptive attribute. As the availability of NH_4^+ in the soil from either soil organic N mineralization or the application of N fertilizers can enhance the activity and populations of nitrifiers, active nitrification can be greatly enhanced (Chu et al. 2008; Chen et al. 2014; Okano et al. 2004). Thus, the regulatory role of NH_4^+ in 1,9-decanediol release can serve an important adaptive function for rice to protect NH_4^+ from nitrification, thereby effecting a higher N-resource utilization efficiency and minimizing N losses from the rhizosphere. This, in turn, carries very important, beneficial environmental consequences in terms of minimizing N pollution and N fertilizer cost (Coskun et al. 2017a, 2017b; Sun et al. 2016b).

In addition, we found that the release of 1,9-decanediol from rice roots can also be enhanced significantly by aeration during root exudate collection (Fig. 4). The likely reason for this is that the release of organic compounds from root cells involves energy-dependent steps (Lalonde et al. 1999). The presence of oxygen can influence the efficiency of ATP production in root cells and is essential to all biosynthetic pathways; in addition, the transport of a very broad range of substrates depends on membrane-bound transport proteins using the energy from ATP hydrolysis (Geigenberger 2003; Weston et al. 2012). Aeration can also improve root vigour and reduce the amount of harmful substances in the root system (Li et al. 2015). Thus, adequate aeration in the trap solutions might enhance root respiration and provide the driving force needed to release 1,9-decanediol from rice roots. Additionally, while rice typically grows in anoxic, NH_4^+-dominated soils (Kronzucker et al. 1998; Kirk and Kronzucker 2005), N mixtures of varying proportions can be expected in soil solution, due to both plant-internal oxygen transport to roots via rice aerenchyma tissue and external oxygen supply, especially in upper soil layers, as the presence of oxygen and co-provision of NO_3^- and NH_4^+ has been shown to facilitate a significant enhancement of growth and yield in rice (Kronzucker et al. 2000). On the one hand, mature rice roots contain large volumes of aerenchyma promoting radial oxygen loss (ROL) from root tissue to the rhizosphere to restrict the accumulation of phytotoxic compounds and maintain aerobic microbial processes, such as the conversion of NH_4^+ to NO_3^-

by nitrifying bacteria (Li et al. 2008). On the other hand, rice roots can release higher quantities of 1,9-decanediol in the presence of oxygen, which, in turn, inhibits active nitrification to achieve an optimization of nitrification rates in the rhizosphere. In general, this regulation of nitrification could be a beneficial strategy for rice to maintain a proper NH_4^+/ NO_3^- level and ratio, thereby reducing N losses and improving NUE, while still benefiting from the presence of small, trace amounts of NO_3^- sufficient for signaling processes (Kronzucker et al. 1995) and optimizing rice growth and yield (Kronzucker et al. 2000).

Our results obtained here indicate that the addition of ammonium-oxidizing bacteria, *Nitrosomonas europaea* and *Nitrosomonas stercoris*, to collection solutions significantly ($P < 0.05$) improved the secretion of 1,9-decanediol from rice roots, whereas the presence of denitrifying bacteria (*Pseudomonas fluorescens 01047* and *RWX31*) made no difference (Fig. 5). Additionally, previous results have shown that rice roots secrete 1,9-decanediol to inhibit nitrification mainly by suppressing the AMO pathway of *Nitrosomonas europaea* (Sun et al. 2016b). Thus, we confirm the important finding that, at the same time as root exudates shape the rhizosphere microbiome, microorganisms influence plant root exudation (Marschner et al. 2001; Matilla et al. 2010; Paterson et al. 2007; Walker et al. 2004). Plants and microbes usually engage in several forms of interaction through secondary metabolites or protein secretions. For instance, alterations in plant amino acid exudation have been observed in the presence of the microbial compounds phenazine, 2,4-diacetylphoroglucinol, and zearalenone (Phillips et al. 2004). Auxin secretion by *Bacillus amyloliquefaciens* FZB42 has been shown to stimulate root exudation in *Triticum aestivum* (Talboys et al. 2014). Both the secretion of seven plant proteins and four proteins of bacterial origin were increased in the *Medicago sativa-Sinorhizobium meliloti* interaction, whereas these proteins were not induced when *M. sativa* was inoculated with *Pseudomonas syringae* DC3000 (De-la-Pena et al. 2008). These findings suggest that secondary metabolites or small molecules may be critical components in the process of signaling and recognition that occurs between roots and soil bacteria. Therefore, we speculate that the interaction between rice roots and these strains of nitrifiers might be mediated through the production of specific chemical signals by *Nitrosomonas*, which could be sensed by rice roots. Rice roots then respond to the presence of ammonium-oxidizing bacteria by increasing the release of 1,9-decanediol to inhibit them in turn, providing the possibility for a feedback loop that can achieve a form of rhizosphere homeostasis in terms of chemical N stability and conversion in the root environment. The differential induction of nitrifiers and denitrifers may indicate that, when rice roots are not perceiving the specific chemical signals, they do not need to stimulate 1,9-decanediol secretion. Ultimately, nitrification in the soil can be subdued efficiently and more nitrogen can be preserved as NH_4^+-N. These results provide important new insight into the complex events that occur in the rice root system to protect NH_4^+ from nitrifying bacteria, in turn optimizing N capture by the rice plant.

Conclusions

Our study presents an analysis of several key factors of both biotic and abiotic nature that govern the release characteristics of 1,9-decanediol, the first BNI identified from rice, the world's most important crop species. Our findings show that the release of 1,9-decanediol can be enhanced by low to intermediate concentrations of NH_4^+ (up to 1.0 mM), and that this is partially due to rhizosphere acidification induced by NH_4^+ uptake and metabolism. We also show that adequate aeration in the rhizosphere is beneficial to 1,9-decanediol exudation. Unlike other BNIs exuded from upland plants (like the pasture grass *Brachiaria humidicola* or *Sorghum bicolor* L.), the induction of 1,9-decanediol from rice by NH_4^+ may be triggered in the whole root system, even when only a localized exposure to NH_4^+ is imposed. More importantly, we show, for the first time, that the secretion of 1,9-decanediol from rice can be induced by the presence of nitrifiers but not denitrifiers. Our ongoing research is aimed at characterization of the chemical signals and mechanisms involved in the interactions between rice and the nitrifying bacteria that reside in its rhizosphere.

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